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DOI: https://doi.org/10.1016/j.tvjl.2014.03.015

Commissioned Review

Laboratory diagnosis of ruminant abortion in Europe

Nicole Borel [a,*], Caroline F. Frey [b], Bruno Gottstein [b], Monika Hilbe [a], Andreas Pospischil [a], Francesca D. Franzoso [a], Andreas Waldvogel [a]

[a] *Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Switzerland*
[b] *Institute of Parasitology, Vetsuisse Faculty, University of Berne, Switzerland*

* Corresponding author. Tel.: +41 446358563.
E-mail address: n.borel@access.uzh.ch

Abstract

Abortion in ruminants is a major cause of economic loss worldwide, and the management and control of outbreaks is important in limiting their spread, and in preventing zoonotic infections. Given that rapid and accurate laboratory diagnosis is central to controlling abortion outbreaks, the submission of tissue samples to laboratories offering the most appropriate tests is essential. Direct antigen and/or DNA detection methods are the currently preferred methods of reaching an aetiological diagnosis, and ideally these results are confirmed by the demonstration of corresponding macroscopic and/or histopathological lesions in the fetus and/or the placenta. However, the costs of laboratory examinations may be considerable and, even under optimal conditions, the percentage of aetiological diagnoses reached can be relatively low. This review focuses on the most commonly occurring and important abortifacient pathogens of ruminant species in Europe highlighting their epizootic and zoonotic potential. The performance characteristics of the various diagnostic methods used, including their specific advantages and limitations, are discussed.

Keywords: Abortion; Infectious; Ruminant; Diagnosis; Zoonotic; Europe

Introduction

An increase in the number of spontaneous abortions in a herd or flock is a dramatic event for the farmer involved, and a range of epizootic and/or zoonotic diseases, or even emerging diseases, may be the cause. In such situations farmers, along with their veterinary practitioners, and potentially state veterinarians, expect rapid reliable results from diagnostic veterinary laboratories, a process that is not always easily achieved. While a plethora of pathogens can cause abortion in ruminants, there is no single diagnostic procedure that can be used to identify these, and in some circumstances the infectious event triggering an abortion may precede it some weeks or months so that evidence of the presence of the pathogen may be obliterated by autolysis. By this time it may no longer be possible to demonstrate a rise in maternal antibody indicative of recent infection. Since attempting to rule out all the possible causes of abortion, can prove costly, diagnostic laboratories primarily focus on the most likely aetiologies and those with zoonotic potential.

This review assesses the most important viral, bacterial, fungal and protozoal causes of abortion in cattle, sheep and goats in an industrialised European country (Switzerland), focusing on the methods used to reach a diagnosis and highlighting protocols that optimise pathogen detection. The information presented will be of interest to laboratory diagnosticians, as well as veterinary practitioners and state veterinarians. An overview of the infectious abortifacients discussed is given in Table 1.

Viral causes

Bovine herpesvirus type I

Bovine herpesvirus 1 (BoHV-1) infections remain a major cause of abortion, venereal and respiratory disease in ruminants in countries where this pathogen has not been eradicated

(Kirkbride, 1992). Latency with recurrent infection is typical for infection with these viruses: during latency the virus survives within cells without causing clinical signs, and upon reactivation, repeated abortion may occur (Nandi et al., 2009). Given that virus is shed during reactivation, an infected animal remains a source of infection for in-contact herd-mates. For this reason, European countries such as Austria, Denmark, Finland, Sweden, Italy, Switzerland and Norway have eradicated this economically significant infection.

BoHV-1 abortion can be diagnosed by demonstrating the presence of the virus in the aborted fetus and, in countries free of the virus, specific antibodies in maternal sera. PCR is currently the most sensitive method of identifying the virus in fetal tissues, particularly the liver (Crook et al., 2012). In endemic regions, serology is of little value in establishing a diagnosis of BHV-1 abortion, as maternal infection may precede abortion for up to two months (Kennedy and Richards, 1964). Thus by the time abortion occurs, maternal antibody levels may have already peaked so that demonstrating a rise in specific antibody levels may no longer be possible. The only grossly visible evidence of BoHV-1 infection in the fetus is subtle multifocal necrosis, particularly of the liver. The fetus is typically autolysed on expulsion with haemoglobin-tinged fluid in its body cavities. Microscopic examination of the liver and adrenal glands may facilitate the identification of necrotic foci with attendant leucocyte infiltration (Schlafer and Miller, 2007). When such lesions are observed, further tests for BoHV-1 infection are recommended, even in regions free of infection.

Pestiviruses

Bovine viral diarrhoea virus (BVDV) and Border Disease virus (BDV) belong to the genus pestivirus of the family *Flaviviridae*, are single-stranded RNA viruses, and exist as both non-cytopathic and cytopathic biotypes, respectively. An animal may remain persistently

infected with a non-cytopathic biotype if exposed during the first trimester of pregnancy (Bachofen et al., 2008; Hilbe et al., 2009). In a retrospective study of bovine abortion in Switzerland between 1986 and 1995, 22/223 (9.9%) were positive for BVDV infection on immunohistochemistry, the second most common cause of infectious abortion after *Neospora caninum* infection (Reitt et al., 2007). Macroscopically visible brain malformations such as porencephaly, hydranencephaly and cerebellar hypoplasia may result from fetal infection (Moening, 1990; Nettleton and Entrican, 1995; Grooms, 2004). However, since such lesions may also result from infection with other viruses, exposure to toxic compounds or genetic disorders, and since fetal infection with BVDV does not necessarily produce morphological alterations, demonstration of the presence of virus is required to confirm the diagnosis.

BDV can cause infertility, abortion, stillbirth and the birth of 'hairy-shaker' lambs depending on the time of fetal infection. The name 'Border Disease' was coined as the disease was first reported in the border region between England and Wales (Sawyer, 1992). In persistently-infected sheep the non-cytopathic virus induces histologically visible myelin deficiency in the CNS, resulting in tremor and an increase or enlargement in the number of primary hair follicles. These 'hairy-shaker' animals are persistently infected with virus (Nettleton, 1987; Sawyer, 1992; Nettleton and Entrican, 1995; Nettleton et al., 1998), and antigen can be found in smooth muscle cells (e.g. of blood vessels), epithelial cells (e.g. of hair root sheath), lymphocytes, neurons and glial cells using immunohistochemistry (Brodersen, 2004; Saliki and Dubovi, 2004; Sandvik, 2005; Hilbe et al., 2007a,b).

Persistent infection with either BVDV or BDV is best confirmed by immunohistochemistry and RT-PCR on skin. Samples from the ear are typically used for this purpose and no fixation or pre-treatment is required. When a whole aborted fetus is available,

104 snap frozen samples of skin, tongue, and thyroid gland are used for immunohistochemistry
105 (Brodersen, 2004; Sandvik, 2005; Hilbe et al., 2007a, b). Carrying out an ELISA on skin or
106 tissue such as thyroid gland from aborted bovine fetuses gives false positive results, perhaps
107 due to the effects of autolysis.
108
109 BVDV or BDV may cross the placenta in persistently infected animals or during the
110 viraemic phase in an acutely infected animal. Infection may go unnoticed as clinical signs
111 may be absent or very mild during acute infection with BVDV and the impact on the fetus
112 depends largely on the stage of fetal development at the time of infection: fetal resorption,
113 abortion, mummification or malformation such as cerebellar hypoplasia or porencephaly may
114 result (Brownlie et al., 1987; Moening, 1990; Nettleton and Entrican, 1995; Grooms, 2004).
115 Infection with non-cytopathic BVDV at approximately 40 to 120 days gestation can induce
116 immunotolerance in the fetus to the infecting virus strain (Brownlie et al., 1987; Brock, 2003;
117 Grooms, 2004; Bachofen et al., 2008), ultimately resulting in persistent viraemia in these
118 animals. By approximately 150 days gestation the fetus is sufficiently immunocompetent to
119 eliminate the infection (Nettleton and Entrican, 1995; Brock, 2003; Grooms, 2004). With
120 BDV ovine fetuses become persistently infected between approximately day 60 and 80 of
121 gestation (Nettleton, 1987; Sawyer, 1992; Braun et al., 2002).
122
123 As abortion due to BVDV infection is often the result of one or more persistently
124 infected animals in the herd, control measures must be directed at identifying and eliminating
125 such individuals (Presi and Heim, 2010; Presi et al., 2011). Since pestiviruses are not strictly
126 species-specific, sheep may infect cattle and vice versa (Carlsson, 1991; Sawyer, 1992; Braun
127 et al., 2002). This factor should therefore be considered in herds where no obvious source of
128 infection can be detected. Zoonotic infections with pestiviruses have not been reported.

Teratogenic viruses

When an increased number of abortions with attendant malformations of the central nervous (CNS) and musculoskeletal systems occur, infection with members of the Orthobunyavirus group or Bluetongue virus (BTV) must be considered. Since these viruses are vector-born their geographic range is limited by the habitat of competent vectors. Nevertheless, both Schmallenberg (SBV) and Bluetongue virus have both recently caused epizooties with consequent massive economic loss in Europe, an area where infections with these viruses had previously been unknown: an important reminder of the importance of the ongoing surveillance of livestock for emerging diseases.

Schmallenberg virus is a novel *Orthobunyavirus*, belonging to the Simbu serogroup of Shamonda/Sathuperi-like viruses. Following its initial detection in dairy cattle in North Rhine-Westphalia in Germany in 2011, this virus infection spread rapidly across Europe: to the Netherlands, Belgium, France, Germany, Italy, Luxembourg, Spain, Switzerland and the UK. Schmallenberg virus RNA was also found in *Culicoides* spp. in Denmark and Belgium (ProMED-Mail, 2012; Rasmussen et al., 2012). Infection may cause hyperthermia, decreased milk production, and watery diarrhoea in adult cattle. Gross lesions in aborted, stillborn and neonatal lambs, goat kids and calves include brachygnatia inferior, torticollis, kyphosis, scoliosis, arthrogryposis, vertebral malformations with unilateral spinal muscle atrophy, hydrancephaly, porencephaly, hydrocephalus, cerebellar hypoplasia and micromyelia (Garigliany et al., 2012; Herder et al., 2012). The main histopathological changes in the CNS are lympho-histiocytic meningoencephalomyelitis with glial nodules in the mesencephalon and hippocampus in lambs and goats, and neuronal degeneration/necrosis in the brain stem of calves. Myofibrillar hypoplasia is also reported in lambs and calves (Herder et al., 2012).

Since infection with other pathogens may result in comparable lesions, infection with SBV requires confirmation by the demonstration of virus in the cerebrum and brain stem, amniotic fluid and/or meconium (OIE, 2012). RT-qPCR is the standard method of detecting SBV in lambs, kids and calves (Garigliany et al., 2012). To date, there is no evidence that SBV is zoonotic (Garigliany et al., 2012). Schmallenberg virus is closely related to Akabane virus (Hahn et al., 2012), and studies of the congenital abnormalities resulting from fetal Akabane virus infection in cattle suggest that hydranencephaly and porencephaly develop when infection occurs between 76 and 104 days of gestation. In contrast, arthrogryposis results from infection at between 103 and 174 days of infection (Kirkland et al., 1988). Evaluating the type of lesions caused by SBV might therefore be useful in estimating the time infection is introduced into a naïve herd. However, more data from field cases and/or experimental infections will be needed in order to fully characterise this recently emerged disease.

Blue tongue virus belongs to the family *Reoviridae*, genus *Orbivirus*, and is usually transmitted to domestic and wild ruminants by haematophagus insects of the genus *Culicoides* (Maclachlan, 2011). The European strain BTV-8, which emerged in the summer of 2006, is one of the most virulent, spreading rapidly through western and central Europe. Infection resulted in significant infertility, early embryonic death, abortions and stillbirth, as well as cerebral malformation (Dal Pozzo et al., 2009; Maclachlan et al., 2009; Wouda et al., 2009; Saegerman et al., 2011; OIE, 2012). Hydrancephaly was described in calves and lambs at necropsy, and brachycephaly with brachygnathia superior and cerebellar aplasia were also found in lambs (Vercauteren et al., 2008; Williamson et al., 2010; Saegerman et al., 2011).

179 The CNS malformations caused by BTV infection have been linked to the particular
180 susceptibility of neuronal and glial progenitor cells to infection prior to their migration to the
181 cerebral cortex and sub-cortical white matter (Maclachlan et al., 2000, 2009). Thus, fetal
182 infection results in cyst formation and dilated ventricles following selective destruction of
183 undifferentiated glial cells (Dal Pozzo et al., 2009). CNS defects in bovine fetuses are most
184 severe if infection occurs prior to 130 days of gestation (Dal Pozzo et al., 2009), and their
185 severity is considered inversely proportional to the period of gestation at which infection
186 occurs (Waldvogel et al., 1992; Maclachlan et al., 2000). The extent of lesions may also be
187 determined by viral dose and virulence and/or by the genetic predisposition of the fetus
188 (Waldvogel et al., 1987; Maclachlan et al., 2009; Williamson et al., 2010). The CNS
189 malformations described in calves infected with serotype-8 in Europe were similar to those
190 reported following infection with serotypes elsewhere (Desmecht et al., 2008; Maclachlan et
191 al., 2009).
192
193 Bluetongue virus is not zoonotic, and a presumptive diagnosis can be confirmed by
194 RT-qPCR on fetal tissues (Toussaint et al., 2007; Vandenbussche et al., 2008). RT-qPCR and
195 sequencing have numerous advantages in terms of speed, sensitivity and specificity and are
196 the most extensively used diagnostic method by reference centres throughout Europe (Brito et
197 al., 2011; Zientara et al., 2012). Other non-viral causes of congenital malformation of the
198 CNS or musculoskeletal system in cattle include plant poisoning (James et al., 1994) and
199 genetic defects (Murphy et al., 2007).
200 **Bacterial causes**
201 *Brucella* spp.
202 Brucellosis causes significant losses due to abortion and infertility in ruminants, but is
203 also zoonotic resulting in persistent or ‘undulant’ fever with influenza-like symptoms and can

204 be fatal when endocarditis and encephalitis ensues (OIE, 2012). The genus *Brucella* contains
205 highly infectious species that infect a wide variety of mammals. *Brucella* spp. are small, non-
206 motile Gram-negative rods, and infection in humans arises from direct or indirect contact with
207 infected animals, or through the consumption of contaminated meat or dairy products (Lista et
208 al., 2011). Percutaneous and airborne infection (in laboratories and abattoirs) have also been
209 documented. Large quantities of bacteria are released into the environment by aborting
210 animals, and animals typically become infected by the oral and venereal routes (Seleem et al.,
211 2010). The genus *Brucella* comprises several species of which *B. melitensis* and *B. abortus*
212 most commonly infect the pregnant uterus of small ruminants and cattle, respectively.
213 *Brucella* spp. considered zoonotic are *B. melitensis*, *B. suis* (biovars 1, 3, and 4), *B. abortus*,
214 and sporadically *B. canis* (Franz et al., 1997; Brenner et al., 2005; Whatmore, 2009). *Brucella*
215 *suis* biovars 2 and 5 are not considered to be pathogenic for humans (Lista et al., 2011).
216
217 Abortion is the most frequent clinical manifestation of brucellosis in ruminants, and
218 grossly visible lesions consist of a leathery thickening of the inter-cotyledonary placenta as
219 well as cotyledonary necrosis (Carvalho Neta et al., 2010). Placental lesions may not be
220 uniformly distributed, and can be difficult to detect in some areas (Carvalho Neta et al.,
221 2010). A presumptive diagnosis is based on the demonstration of organisms using a modified
222 acid-fast stain (STAMP) on placental smears (OIE, 2009). However, other organisms causing
223 abortion in ruminants such as *Chlamydia* spp. and *Coxiella* spp. may also stain with this
224 technique. Confirmation therefore requires demonstration of the agent within the placenta or
225 fetal organs by culture or PCR (Carvalho Neta et al., 2010). Although serology may confirm a
226 diagnosis at a herd level, false positive reactions due to infection with *Yersinia* spp. can occur
227 (McGiven et al., 2009). Since bacterial shedding can extend over long periods of time,

infected animals should be identified and eliminated from the herd as soon as possible in order to minimise the spread of infection.

Chlamydia abortus and *Chlamydia*-like bacteria

Ovine enzootic abortion (OEA) or enzootic abortion of ewes (EAE) is an economically significant disease causing late-term abortions, neonatal losses and the birth of weak lambs. In Europe, OEA is the most common infectious cause of abortion in sheep and goats (Longbottom and Coulter, 2003; Aitken and Longbottom, 2007). The same organism can also infect cattle, but attendant abortion is more sporadic (Borel et al., 2006). OEA is caused by infection with the Gram-negative, obligate intracellular bacterium *Chlamydia abortus* of the *Chlamydiaceae* family within the order *Chlamydiales*. To date, the order *Chlamydiales* consists of at least eight families (Wheelhouse and Longbottom, 2012).

The seven recently described families have been termed *Chlamydia*-like or *Chlamydia*-related bacteria. Each of these families exhibits an 80-90% 16S rDNA sequence similarity to the family *Chlamydiaceae* (Wheelhouse and Longbottom, 2012). Within *Chlamydia*-like bacteria, *Waddlia chondrophila* (family *Waddliaceae*) was first isolated from an aborted bovine fetus in the USA (Dilbeck et al., 1990). Members of the *Chlamydia*-related families *Parachlamydiaceae* and *Rhabdochlamydiaceae* have been recently implicated as novel abortifacients in cattle, and to a lesser extent small ruminants in Switzerland and the UK (Borel et al., 2010; Deuchande et al., 2010; Wheelhouse et al., 2010b; Wheelhouse and Longbottom, 2012).

OEA is typically introduced into an immunologically-naïve flock by a sub-clinically infected pregnant ewe. Clinical signs in infected pregnant sheep are rarely observed and are

253 limited to vaginal discharges, seen more frequently in goats than sheep. Abortion occurs in
254 the last 2-3 weeks of gestation or weak/dead lambs are delivered at term, both accompanied
255 by the shedding of very large numbers of infectious organisms. Placentitis is key to the
256 pathogenesis of chlamydial abortion (Buxton et al., 2002): placental infection becomes
257 established between day 60 and 90 of gestation, with lesions first detectable after 90 days.
258 Infection of the fetus is secondary to placentitis of probable embolic origin. Where non-
259 pregnant females or females in late pregnancy become infected, infection remains latent at a
260 currently undetermined anatomical location and may induce abortion in the next gestation
261 (Pospischil et al., 2010).
262
263 Placental membranes and cotyledons are the specimens of choice for diagnosing
264 chlamydial abortion due to *C. abortus* as these contain myriad organisms. Alternatively,
265 swabs from the vagina at the time of abortion or of the moist coat of aborted fetuses can be
266 used (Longbottom and Coulter, 2003). Historically, isolation in cell culture was considered
267 the 'gold standard' test but is time-consuming and expensive, and requires specialist
268 expertise. A presumptive diagnosis of chlamydial abortion is made when late-term abortions
269 occur and when the placenta exhibits macroscopically visible oedematous, inflammatory and
270 necrotic lesions covered by purulent exudate. Smears prepared from the placental membranes
271 and cotyledons can be stained using the modified Ziehl-Neelsen, Giemsa, Gimenez, or
272 Machiavello techniques in order to identify chlamydial elementary bodies (EBs) (Sachse et
273 al., 2009). Although the sensitivity and specificity of stained smears are somewhat
274 insufficient, these can be used as an initial screen when PCR methods are not available.
275 Histopathologically, purulent to necrotising placentitis with vasculitis are features, and if
276 attendant thrombo-embolic infection of the fetus occurs, necrotic and inflammatory lesions
277 are observed in the lung and liver (Buxton et al., 2002). Placental or fetal tissues can be

examined by immunofluorescence and immunohistochemistry, using antibodies directed against the chlamydial lipopolysaccharide (LPS) or other surface antigens (Sachse et al., 2009). However, such antibodies are often not species-specific.

Currently, nucleic acid amplification tests (NAATs) are considered the gold standard for the diagnosis of chlamydial infections in humans and animals: conventional and real-time PCR methods specific for *C. abortus*, and *Chlamydiaceae* family-specific PCR assays. Such *Chlamydiaceae* family-specific PCR protocols are often used as a screening method to be followed by chlamydial species determination, using a species-specific PCR (Pantchev et al., 2010), by sequencing of the PCR product, or by ArrayTube microarray (Borel et al., 2008). Contamination of the placenta and expelled fetus with bedding and faecal material at parturition/abortion can lead to false-positive PCR results. Correlation of the typical histopathology with detection of the agent or its DNA in lesions is required to unambiguously confirm a diagnosis (Borel et al., 2006; Sachse et al., 2009). Methods recommended for diagnosing chlamydial abortion in cattle are similar to those used in sheep and goats. However, bovine chlamydial abortion is more sporadic and the placental antigen load is substantially less than in sheep and goats (Borel et al., 2006). The serological diagnosis of chlamydial abortion is limited by several factors and is not recommended on an individual animal basis: none of the currently available serological tests are able to differentiate vaccinated from naturally-infected animals.

Samples from ruminant abortions are not routinely screened for *Chlamydia*-like organisms and *Chlamydiaceae*-family-specific PCR methods are not capable of amplifying *Chlamydia*-like organisms. These infections are frequently detected by nucleic acid amplification assays targeting highly conserved genes such as the 16S rRNA gene, a step

303 which is then followed by sequencing (Corsaro and Greub, 2006; Lienard et al., 2011).
304 Specific real-time PCRs to detect *Parachlamydia* (Casson et al., 2008) and *Waddlia*
305 *chondrophila* (Goy et al., 2009) in samples of placenta and aborted fetus from cattle (Ruhl et
306 al., 2009; Blumer et al., 2011) and sheep/goats (Ruhl et al., 2008) have been described. In
307 cases due to *Parachlamydia*, the intracellular bacteria and inclusions may be detectable in
308 haematoxylin and eosin-stained formalin-fixed, paraffin-embedded samples of cotyledon
309 (Wheelhouse et al., 2012). Histopathologically, a purulent and/or necrotising placentitis is the
310 most consistent finding with *Parachlamydia* infection (Borel et al., 2010; Wheelhouse et al.,
311 2012).
312
313 *Chlamydia*-like organisms may play a role in bovine and, to a lesser extent,
314 ovine/caprine abortion. However, more data is needed to elucidate the host range, pathogenic
315 potential and transmission routes of this pathogen in farm animals. Where inflammation of
316 undetermined aetiology is identified in samples, these should be forwarded to specialised
317 laboratories for further investigation. Abortion due to *C. abortus* or *Chlamydia*-like organisms
318 emphasise the importance of including placental samples when examining ruminant abortion
319 material (Deuchande et al., 2010).
320
321 Control strategies for OEA include antibiotic treatment and vaccination. Currently,
322 inactivated and modified live vaccines are available (Longbottom and Livingstone, 2006).
323 The live vaccine contains the 1B mutant strain of *C. abortus* originally derived from the
324 virulent field strain AB7 and represents a temperature-sensitive variant (Rodolakis, 1983).
325 Comparison of the parental and mutant strain identified 22 single nucleotide polymorphisms
326 (SNPs) unique to the mutant (Burall et al., 2009). There is some evidence that the 1B vaccine
327 strain could cause OEA (Wheelhouse et al., 2010a), and PCR-RFLP markers allow the

differentiation between *C. abortus* field strains and the 1B live vaccine strain (Laroucau et al., 2010). The same differentiation is possible using a novel and less time-consuming high-resolution melt PCR analysis (Vorimore et al., 2012). *Chlamydia abortus* is zoonotic and poses a considerable risk of abortion in pregnant women (Buxton, 1986; Pospischil et al., 2002). *Waddlia chondrophila, P. acanthamoebae* and *Rhabdochlamydiaceae* may also have zoonotic potential as all have been associated with recurrent pregnancy failure (Baud et al., 2008) and miscarriage (Baud et al., 2011), as well as with pneumonia (Greub, 2009) and bronchiolitis in children (Goy et al., 2009).

Coxiella burnetii

Coxiella burnetii is a gram-negative, intracellular bacterium with worldwide distribution. In ruminants, *C. burnetii* infection can result in late-term abortion, stillbirth, and in the delivery of weak/non-viable neonates (Palmer et al., 1983; Moore et al., 1991; Bildfell et al., 2000). In non-pregnant animals, infection is typically subclinical with reactivation occuring during pregnancy. The organisms are excreted in large quantities at parturition in placental, vaginal and uterine discharges, as well as in milk, urine, and faeces (Woldehiwet, 2004). Transmission is largely by the inhalation of aerosols from the infected placenta, body fluids or contaminated dust following desiccation of exudates (Angelakis and Raoult, 2010). *Coxiella burnetii* can survive in the environment long-term as an endospore-like structure, and can exist in two antigenic phases: a virulent, naturally occurring phase one form and a less virulent phase II form which develops when the organism is cultured in vitro (Quevedo Diaz and Lukacova, 1998).

Q (for query) fever in humans caused by *C. burnetii* infection, is a widely occurring zoonosis (Rodolakis, 2009), its zoonotic potential recently highlighted by a severe outbreak of

353 disease in the Netherlands. Aborting domestic ruminants are the main source of human
354 infection, and it is largely transmitted through inhalation of aerosols (Maurin and Raoult,
355 1999; Woldehiwet, 2004; Angelakis and Raoult, 2010). The placenta and amniotic fluids are
356 the samples of choice for detecting *C. burnetii* in aborting ruminants: the organism can be
357 demonstrated in impression smears from such samples stained using the Machiavello or
358 modified Ziehl-Neelsen techniques. Given that *C. burnetii* must be differentiated
359 morphologically from *C. abortus* or *B. abortus*, these identification methods have limited
360 specificity and inferior sensitivity to PCR techniques (Woldehiwet, 2004; Arricau-Bouvery
361 and Rodolakis, 2005). PCR can be performed on placental samples, genital swabs, milk, or
362 faeces, and a real-time PCR targeting the insertion sequence IS111 gene of *C. burnetii* was
363 found to be highly sensitive (detection limit of 10 copies of template), and specific when
364 tested on ruminant placental cotyledons and fetal samples (Jones et al., 2010).
365
366 Demonstrating the presence of *C. burnetii* does not necessarily mean this organism
367 caused the abortion, as clinically normal animals can shed bacteria. Therefore, identifying
368 macroscopically and microscopically visible supportive lesions in the placenta is important:
369 goats and sheep exhibit intercotyledonary thickening and exudation. Acute suppurative and
370 necrotising placentitis often without vasculitis is present histologically, and cytoplasmic
371 vacuoles in chorionic epithelial cells contain large numbers of organisms in well-preserved
372 specimens (Moore et al., 1991; Sachez et al., 2006). In cases of bovine abortion, the affected
373 chorionic stroma is infiltrated by mononuclear cells, and there is accompanying necrosis of
374 chorionic trophoblasts, and focal exudation of fibrin and neutrophils (Bildfell et al., 2000).
375 Serology is not useful in diagnosing abortions in individual ruminants as animals can shed
376 bacteria prior to seroconversion, while others never seroconvert despite the fact that they have

377 been infected and shed *C. burnetii* over a long time period (Berri et al., 2002; 2007; Rousset
378 et al., 2009).
379
380 Given that massive amounts of infectious material is shed at the time of abortion
381 caused by *C. burnetii*, it is essential that environmental contamination is limited through the
382 appropriate disposal of placental and fetal material, cleansing and disinfection, and the
383 separation of pregnant from aborting animals. Animals that are aborting and those in late
384 pregnancy can be treated with tetracyclines, although this will not fully prevent abortions or
385 the shedding of *C. burnetii* (Rodolakis, 2009; Stuen and Longbottom, 2011). Vaccines are
386 available but their production has been hampered by the 'phase variation' exhibited by *C.*
387 *burnetii*. Phase I vaccines can prevent abortion and reduce shedding of *C. burnetii* thus
388 reducing the risk of dissemination but these do not eliminate *C. burnetii* from animals
389 naturally infected prior to vaccination (Arricau-Bouvery et al., 2005; Arricau-Bouvery and
390 Rodolakis, 2005; Stuen and Longbottom, 2011).
391
392 *Salmonella* Abortusovis
393 Infection with *Salmonella enterica* subsp. *enterica* serovar Abortusovis *(Salmonella*
394 Abortusovis), is highly specific for sheep: human infection has not been reported (Jack,
395 1968). In a naïve herd, this pathogen can cause abortion during the last trimester in 30–50%
396 of pregnant ewes. In endemic regions, infection may only result in sporadic abortions as a
397 result of background protective immunity (Wirz-Dittus et al. 2010). Aborted fetuses are
398 typically severely autolysed, and fetal lesions are relatively non-specific. Diagnosis is based
399 on the demonstration of the pathogen within the fetus, in particular in the stomach contents,
400 and in this context PCR is more sensitive than culture (Belloy et al., 2009).

Infections with *Salmonella* Abortusovis are quite common in Switzerland, although cases are rarely reported, despite the fact that infection is notifiable (Wirz-Dittus et al., 2010). This is likely due to the fact that there is insufficient awareness of and/or inadequate diagnostic techniques to detect, this infection. While most bacteria causing fetal infections can be cultured on blood and Mac Conkey agar after aerobic incubation for 24 - 48 h, *Salmonella* Abortusovis may not be detected for 48 h (P. Boujon, personal communication). Furthermore, *E. coli*, a frequent contaminant in abortion samples, may inhibit the growth of *Salmonella* Abortusovis (Belloy et al., 2009). Thus PCR is the method of choice for detecting *Salmonella* Abortusovis.

Miscellaneous bacteria

Diverse bacterial species, of varying pathogenicity, are associated with sporadic abortions in ruminants. Many of these bacteria are ubiquitous and are facultative pathogens not typically associated with disease in adult animals: they are often found in the environment or on mucous membranes. A maternal bacteraemia is considered the most common pathway through which bacteria reach the gravid uterus and infect the placenta/fetus. The resulting abortions may occur at any stage of gestation, but occur most commonly in the second and last trimesters. Stillbirth and birth of weak, non-viable neonates may also occur.

A major challenge in the diagnosis of bacterial abortion is distinguishing environmental contamination of the fetus from true fetal infection (Kirkbride, 1990). The following three criteria have been proposed to determine if bacteria, identified in an aborted fetus, are significant:

(i) The organism is found in large numbers and/or pure or almost pure culture in the fetal abomasum and/or other tissues.

(ii) There is associated inflammation in the fetal tissues and/or membranes.

(iii) Tests exclude other common abortigenic agents.

If all three criteria are met, the causative role of the bacterial species involved can be established with significant certainty. However, in some cases a rapid onset, lethal bacterial septicaemia in the fetus may result in abortion before it can mount a discernible inflammatory response.

Most of the bacteria associated with abortion in ruminants can be isolated from the placenta, fetal organs (such as lung and liver), and abomasal contents by aerobic culture on standard media such as sheep blood and bromothymol-blue lactose agar. Anaerobic bacteria may be under-reported as abortifacients as anaerobic culture is not usually carried out as part of routine diagnostic procedures. Gram staining of smears from placental membranes or abomasal fluid may facilitate the detection of *Trueperella pyogenes*, while specialised conditions are required to culture *Ureaplasma* spp., *Mycoplasma* spp. and *Campylobacter* spp. from cases of bovine abortion.

Multifocal hepatic necrosis and suppurative bronchopneumonia are lesions typically identified in fetuses infected with bacteria. Fetal icterus may suggest leptospiral infection, but confirming this diagnosis can be challenging given that *Leptospira* spp. are very labile and difficult to culture: a mild lymphoplasmacytic interstitial nephritis may be present on histopathological examination and the characteristic long slender bacteria may be demonstrated by Warthin-Starry staining. Examination of fetal kidney impression smears using a fluorescent antibody (FA) test with multivalent antisera is convenient, rapid and inexpensive but not particularly sensitive and specific diagnostic method.

Listeria monocytogenes and possibly *L. ivanovii* may cause sporadic abortions at all stages of pregnancy, as well as stillbirths and neonatal septicemias in sheep, cattle and goats (Dennis, 1975; Moeller, 2001). Following abortion, the placenta is often retained and the fetus exhibits marked autolysis. In this case the bacteria access the fetal bloodstream via the placenta, leading to generalised infection with attendant miliary pyogranulomatous lesions mainly in liver and placenta (Low and Donachie, 1997; Vazquez-Boland et al., 2001). *Listeria* spp. are readily isolated from fetal abomasal content and placenta (Kirkbride, 1990; Njaa, 2012), and antigen can be detected in formalin-fixed, paraffin-embedded tissues using immunohistochemistry (Navarro et al., 2009).

Campylobacter fetus ssp. *fetus* is found in the intestine of cattle and sheep and while the organism can cause sporadic bovine abortion, this event more commonly occurs in sheep (Moeller, 2001; Schlafer and Miller, 2007). Sources of infection include feed or water contaminated with faeces, and infected exudates around parturition. Grossly visible fibrinous peritonitis and/or pleuritis/pericarditis with accompanying large circumscribed foci of hepatic necrosis, are typical findings (Schlafer and Miller, 2007). Placental lesions consist of necrosuppurative inflammation with vasculitis and trophoblasts containing numerous small gram-negative bacteria (Kirkbride, 1993a,b; Schlafer and Miller, 2007). Special media are required to isolate this bacterium (OIE, 2012).

Parasitic causes

Neospora caninum

Neospora caninum is one of the most important causes of infectious abortion in dairy cattle in industrialised countries, with a prevalence rate of up to 40% (Dubey and Schares, 2011). Abortions due to *N. caninum* have also been reported for small ruminants (Haessig et

476 al., 2003; 2011). This protozoan parasite is taxonomically located within the family
477 *Toxoplasmatidae* and the order *Eimeriina.* It is closely related to *Toxoplasma gondii*, and
478 dogs, wolves, coyotes and dingoes have been identified as final hosts (Dubey and Schares,
479 2011). Environmentally-resistant oocysts are shed in the faeces and intermediate hosts may
480 become infected by the oral route. Most importantly, *N. caninum* infection can persist in cows
481 and cause fetal infections over several pregnancies (Davison et al., 1999). Infected animals
482 may abort or deliver weak or clinically normal congenitally-infected calves. In cattle, vertical
483 infection occurs approximately ten times more frequently than oral infection (Bartels et al.,
484 2007; Dijkstra et al., 2008), although the latter mode of infection may cause substantial
485 abortion 'storms' (Sager et al., 2005).
486
487 There are no specific gross lesions in aborted fetuses with a diagnosis resting on the
488 demonstration of small disseminated foci of necrosis by histopathological examination and
489 examination of brain tissue by PCR (Baszler et al., 1999). Although PCR is more sensitive
490 than histopathology, the characteristic microscopic lesions in the brain, skeletal muscle, heart
491 and placenta are required to confirm the diagnosis (Dubey and Schares, 2006): these lesions
492 consist of necrotic foci and mononuclear cell infiltrates (Schlafer and Miller, 2007).
493 Immunohistochemistry can also be used to demonstrate parasitic antigen in fetal tissues.
494 Seropositive cows are more likely to abort than seronegative animals and this risk increases
495 with increasing levels of *N. caninum*-specific antibodies in individual animals. Thus *N.*
496 *caninum*-specific antibody concentrations may be useful in identifying 'at risk' animals
497 (Dubey et al., 2007). Antibody avidity correlates with stage of infection, thus allowing
498 serological discrimination between recently and chronically infected animals (Sager et al.,
499 2003). Neosporosis is primarily a disease of cattle and is not considered zoonotic (Dubey and
500 Schares, 2011).

Toxoplasma gondii

Toxoplasma gondii infection is a frequent cause of abortion in small ruminants: Chanton-Greutmann et al. (2002) identified this parasite as the cause of abortion in 19% and 15% of cases in sheep and goats, respectively. Toxoplasmosis is considered one of the most significant food-borne zoonoses worldwide (EFSA, 2007), and the life cycle of the organism includes a feline definitive host that can excrete environmentally-resistant oocysts, and several vertebrate intermediate hosts, including wildlife species. The route of infection for both definitive and intermediate hosts is oral or transplacental (Dubey, 2009). While no significant grossly visible lesions are evident in aborted fetuses, numerous white foci 1 – 3 mm in size are present on placental cotyledons and the intercotyledonary tissue exhibits oedema (Schlafer and Miller, 2007). Histologically, necrotic foci are observed in the white matter of the brain and in the cotyledons (Schlafer and Miller, 2007).

To incriminate toxoplasmosis as a cause of abortion, fetal fluids can be indirectly assessed for parasite-specific antibodies, a technique that can be complemented by the direct detection of parasite-antigen in brain and/or placenta (Uggla et al., 1987; Dubey, 2009). As described above for *N. caninum*, histopathology complemented by immunohistochemistry can be carried out on fetal tissues. Furthermore, highly sensitive, specific PCR diagnostic techniques exist (Switaj et al., 2005). In contrast to *N. caninum, T. gondii* rarely causes repeated abortions (Dubey, 2009).

Tritrichomonas foetus

Tritrichomonosis is a specific contagious disease of cattle associated with abortion, or more often, early embryonic death or infertility. *Tritrichomonas foetus* is venereally

transmitted, and sub-clinically infected bulls may play an important role in the spread of infection. Although effectively controlled by the use of artificial insemination, the disease remains a major cause of reproductive loss where natural breeding is practiced: e.g. in large beef herds in the Americas, South Africa and Australia (Campero and Gottstein, 2007). No intermediate hosts or environmentally stable forms are known for *T. foetus* and no specific lesions are induced in the fetus. However, large numbers of the protozoa may be found in the fetal fluids and stomach (Schlafer and Miller, 2007). The most valuable samples useful in diagnosing abortion due to this parasite are fetal abomasal fluid, followed by other fetal fluids, and the organism can be demonstrated by PCR (Felleisen, 1997; BonDurant et al., 2003; Grahn et al., 2005) or culture. The parasites may also be visible by histopathology and/or immunohistochemistry on aborted fetal tissue or placenta (Rhyan et al., 1988; 1995). Although described with tritrichomonosis, pyometra is a relatively uncommon complication (Schlafer and Miller, 2007). Control measures include the survey of all animals in a herd, especially bulls, for freedom of infection, and the culling of infected animals identified.

Fungal causes

Aspergillus spp.

Mycotic abortions in ruminants are usually sporadic events. Knudtson and Kirkbride (1992) evaluated the results of the laboratory diagnoses of 6,858 cases of bovine abortion and stillbirth in the northern plains region of the USA: they identified fungi as the cause of abortion in 6.8% of cases with *Aspergillus fumigatus* most frequently incriminated (5%). This fungal species was also determined to be the most common mycotic cause of abortion in Denmark (Jensen et al., 1991) and Ireland (Sheridan et al., 1985). However other species of saprophytic/environmental fungi such as *Absidia* spp., *Mucor* spp., *Rhizopus* spp. and *Candida* spp. have also been implicated in fetal loss (Schlafer and Miller, 2007).

Mycotic abortions usually occur in the third trimester of pregnancy, and clinical signs in the dam are infrequently observed apart from retention of the placenta (Schlafer and Miller, 2007). The diagnosis of fungal abortion is based on the demonstration of the typical grossly and histopathologically visible lesions in association with the presence of fungi. Grossly visible placental lesions include a leathery, diffusely thickened intercotyledonary placenta with necrotic haemorrhagic infarcts in the cotyledons. Fetal lesions may be absent and autolysis minimal. Occasionally, locally extensive circular skin lesions may be present on the fetus. Microscopically there is typically a severe suppurative placentitis and vasculitis with intralesional fungi (Schlafer and Miller, 2007). Occasionally, inflammatory lesions associated with fungal invasion may be present in the fetal respiratory or digestive tracts. Direct identification of the fungi using a potassium hydroxide wet-mount examination of lesion scrapings may facilitate the diagnosis. The observation of yeast-like and hyphal elements on histopathological examination of the placenta may be facilitated by the use of periodic acid-schiff (PAS) and Grocott's methenamine silver stains (Jensen et al., 1991). Since abortifacient fungi may be present in the environment as saprophytes, their recovery from placenta on culture can lead to false positive diagnoses, and culture of lung or intestinal samples are considered more reliable. The fertility of cows does not appear to be impaired by fungal abortion (McCausland et al., 1987).

Discussion

The objective of this review article is to provide essential information about the most commonly occurring causes of ruminant abortion in Europe. Veterinary laboratory diagnosticians need to provide expert advice to veterinary practitioners and state veterinarians on the taking of the most appropriate samples in cases of ruminant abortion as well as on the interpretation of test results. Sourcing the relevant information can prove challenging as such

material is often dispersed over a wide variety of sources. In many instances, the only samples submitted to a diagnostic laboratory are fragments of fetal membrane, maternal blood samples, and a very rudimentary clinical history. Such samples may not be appropriate when attempting to identify a number of the abortigenic pathogens we have discussed in this review. Determining the cause of an abortion must thus start with the correct sampling protocol by the veterinarian 'in the field'.

In terms of case history, information indicating the number of recent and previous abortions, the number of pregnant animals, and the time-period since the first abortion should be identified, as well as details of any fetal malformations. Such information may be important in supporting/validating the subsequent laboratory results or otherwise. For example, while fungal infection is a plausible explanation for sporadic abortions, it is unlikely to result in a full scale abortion 'storm'.

Retention of fetal membranes may make it difficult to obtain sufficient sample material, and placenta may also be difficult to locate in animals aborting at pasture. Nonetheless, the sampling and testing of placenta is central to the diagnosis of *Brucella* spp., *Chlamydia* spp., *Coxiella burnetii*, and *Toxoplasma gondii*. Since these pathogens are also potentially zoonotic, at least three cotyledons and some intercotyledonary placenta should be sampled. Where possible, the entire fetus should be submitted, especially when a bacterial cause is suspected as *Salmonella* Abortusovis may not grow in the presence of contaminating *E. coli*. Aborted material needs to be treated as a biohazard and any contamination of the environment minimized through the use of waterproof containers and surrounding absorbent material. Although large, full-term bovine fetuses may prove challenging in terms of their

size, it is recommended that at least the head be submitted given the requirement to examine the brain in reaching a diagnosis of the important bovine abortifacient *N. caninum*.

At the diagnostic laboratory, a step-wise investigative approach is recommended. The following diagnostic approach is illustrated by the associated figures:

i) Obtain relevant case history: early vs. late-term abortion, mummified vs. fresh fetus, number of abortions in relation to number of pregnant animals, time interval from first abortion to case submission and corresponding number of abortions, clinical signs in dams, changes in husbandry, transport over long distances.

ii) Collect blood from affected animals for serology: useful in diagnosing non-endemic infections and/or infections against which animals are not vaccinated.

iii) Carry out a macroscopic examination of the placenta (Figs. 1 and 2) and fetus (Figs. 3-5).

iv) Sample placenta and fetal organs for microbiology, histopathology, and molecular analysis. Sampling fetal body fluids may facilitate the diagnosis of viral infections with BTV or Schmallenberg virus.

v) Perform routine bacteriology including direct smears, aerobic culture and the digestion of suspicious lesions with potassium hydroxide to demonstrate potential fungal organisms.

vi) Carry out histopathological examination of the placenta and/or fetal organs: valuable in distinguishing bacterial contamination from infection (Figs. 6-8).

vii) Use immunohistochemistry or in situ hybridisation to demonstrate presence of pathogen within placental/fetal tissue (Figs. 9 and 10).

viii) Notifiable diseases reported on diagnosis.

Where gritty, chalk-like depositions in cotyledons are confirmed on histopathological examination as metastatic mineralisation, a visit to the farm of origin would be required to assess if feed or pastures are contaminated by calcinogenic plants such as *Trisetum flavecens*. Serology may yield valuable information about the health status of a herd in areas where diseases such as brucellosis, or in some countries bovine herpes virus -1 infection, have been eradicated. However, in areas where these infectious agents are endemic the diagnostic value of a single serum sample from a dam may be limited. Seropositivity may be the result of vaccination against, or exposure to, the pathogen. Furthermore, paired serum samples to demonstrate seroconversion at the time of abortion may not yield a conclusive result either since maternal infection may precede abortion by several weeks, i.e. the antibody titer may no longer be rising at the time of abortion. Serology on fetal heart blood or on thoracic/abdominal fluid can prove useful in diagnosing infection with viruses such as BDV, Schmallenberg and bluetongue virus, other *Bunyaviridae* and *Flaviviridae* (Njaa, 2012).

The application of molecular-based PCR-type techniques is becoming more commonplace in veterinary diagnostic laboratories. Although these tests are often highly sensitive and specific, particular technical requirements, expertise and additional costs can restrict their application. Ultimately, the linking of molecular findings to grossly and/or microscopically visible lesions is always recommended in order to mitigate against false-positive results.

Conclusions

In summary, the accurate diagnosis of abortion in ruminants (or indeed other species) needs to be based on: obtaining a concise clinical history, careful gross and histopathological examination of any placental/fetal lesions, and the selection and submission to the laboratory of appropriate samples. The results of the tests carried out should be correlated back to the

particular circumstances of the abortion problem in order to validate their plausibility. While such stepwise, methodical procedures may not necessarily raise the percentage of fetal laboratory submissions where an exact diagnosis is reached, they will facilitate the out-ruling of many infections of epizootic and zoonotic importance.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

The authors wish to thank Professor M.M. Wittenbrink, of the Institute for Veterinary Bacteriology, Vetsuisse Faculty, at the University of Zurich for his helpful input und discussion.

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Table 1

Overview of infectious causes of abortion in cattle, sheep and goats in Europe.

Infectious agent		Cattle	Sheep	Goats
Viruses	Bovine herpesvirus type-1	++, dt, epi	-	-
	Pestiviruses	++ [a], dt, epi and vt	+ [b], dt, epi and vt	-
	Teratogenic viruses:			
	Bluetongue virus	+, vb, enz	+, vb, enz	+, vb, enz
	Schmallenberg virus	++, vb, enz	++, vb, enz	++, vb, enz
Bacteria	*Brucella* spp.	++, dt, epi, zoo	++, dt, epi, zoo	++, dt, epi, zoo
	Chlamydia abortus	+, dt, epi, zoo	++, dt, epi, zoo	++, dt, epi, zoo
	Coxiella burnetii	++, dt, epi, zoo	++, dt, epi, zoo	++, dt, epi, zoo
	Salmonella Abortusovis	-	++, dt, epi	-
	Miscellaneous bacteria	+	+	+
Parasites	*Neospora caninum*	++, ih and vt	+, ih	+, ih
	Toxoplasma gondii	-	++, ih	++, ih
	Tritrichomonas fetus	+	-	-
Fungi	*Aspergillus fumigatus*	+	+	+

++, important in this species; +, occasional cause in this species; -, of unknown significance in this species.
epi, epizootic; enz, enzootic; zoo, zoonotic; vb, vector borne; dt, direct transmission; ih, intermediate host; vt, vertical transmission
[a] Bovine viral diarrhoea virus
[b] Border disease virus

Figure legends

Fig. 1. Photograph of normal bovine placenta illustrating a cotyledon and surrounding inter-cotyledonary tissue.

Fig. 2. Photograph illustrating a purulent and necrotising placentitis of unknown aetiology. A placental cotyledon exhibits diffuse reddening and multifocal yellow discolouration (necrosis). The inter-cotyledonary tissue is covered in purulent exudate.

Fig. 3. An aborted ovine fetus showing arthrogryposis and scoliosis. Schmallenberg virus RNA was detected in the fetal brain by RT-PCR.

Fig. 4. An aborted bovine fetus exhibiting multifocal fungal plaques over the head, left ear and neck region.

Fig.5. Brain of a neonatal calf with cerebellar hypoplasia as a result of in-utero bovine viral diarrhoea virus infection.

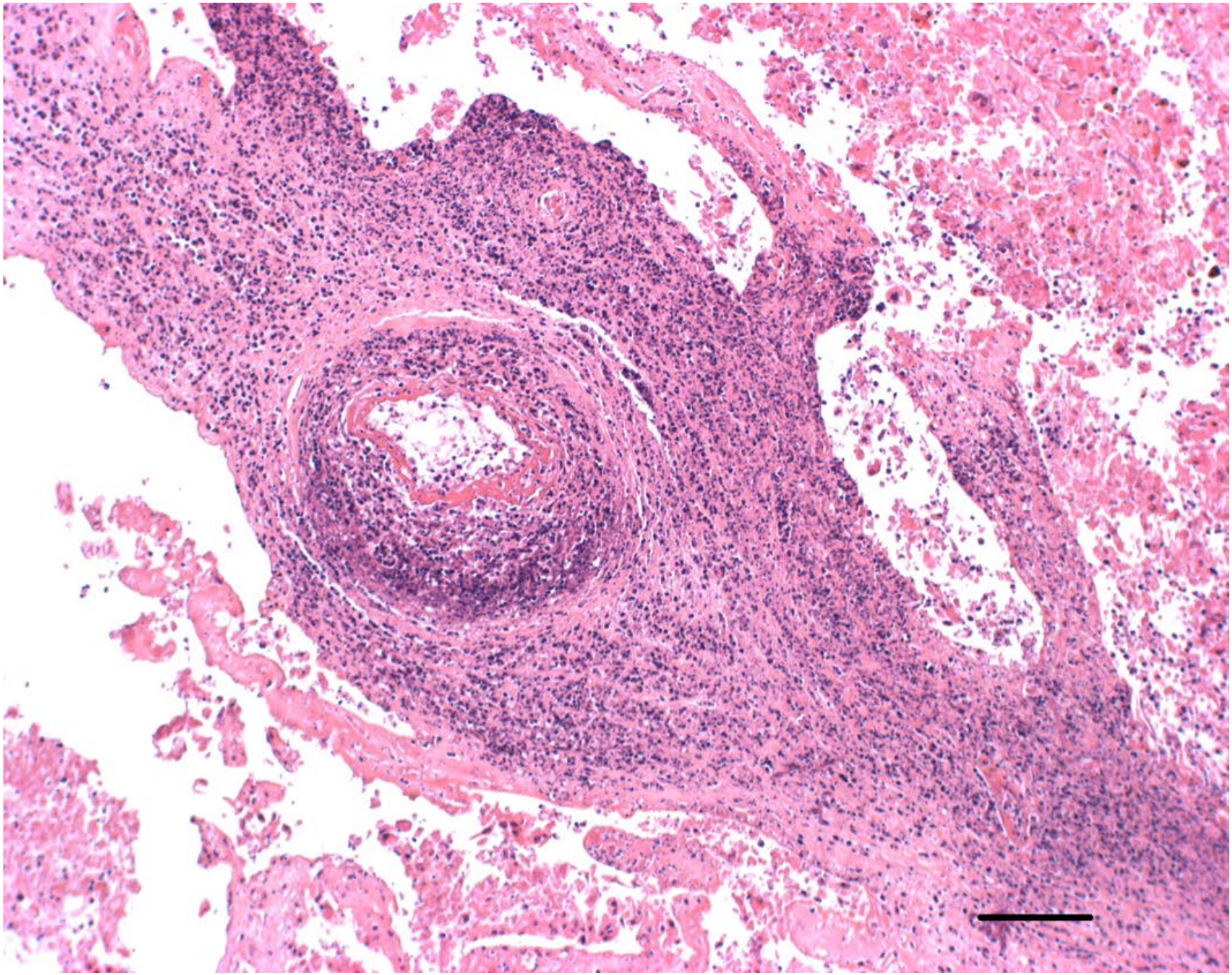
Fig. 6. Photomicrograph of bovine placenta illustrating a purulent and necrotizing placentitis with vasculitis of a placental arteriole (arrow). Haematoxylin and eosin stain. Scale bar, 100 μm.

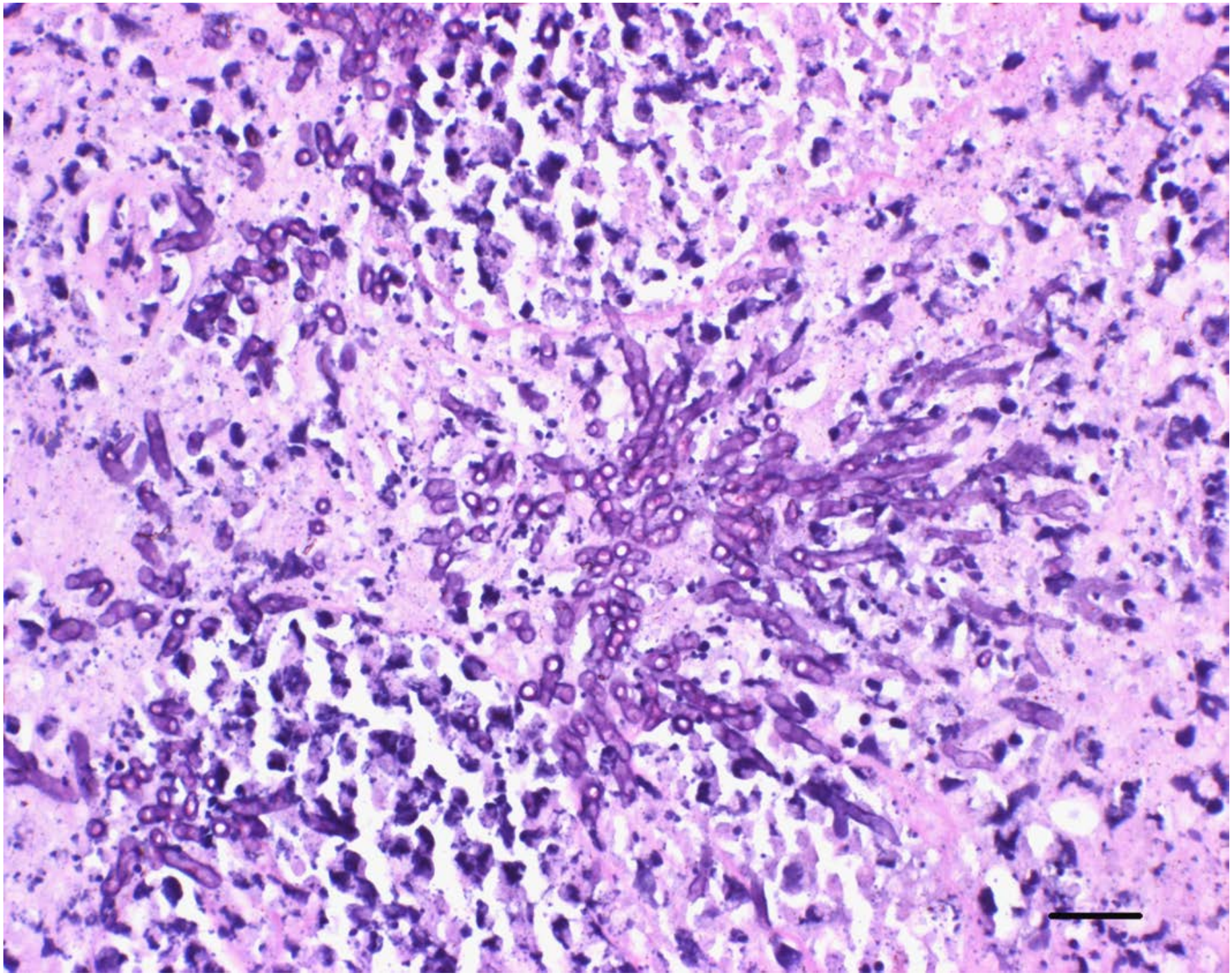
Fig. 7. Higher magnification photomicrograph of placenta illustrated in Fig. 6 illustrating myriad PAS-positive fungal hyphae. Periodic acid schiff stain. Scale bar, 20 μm.

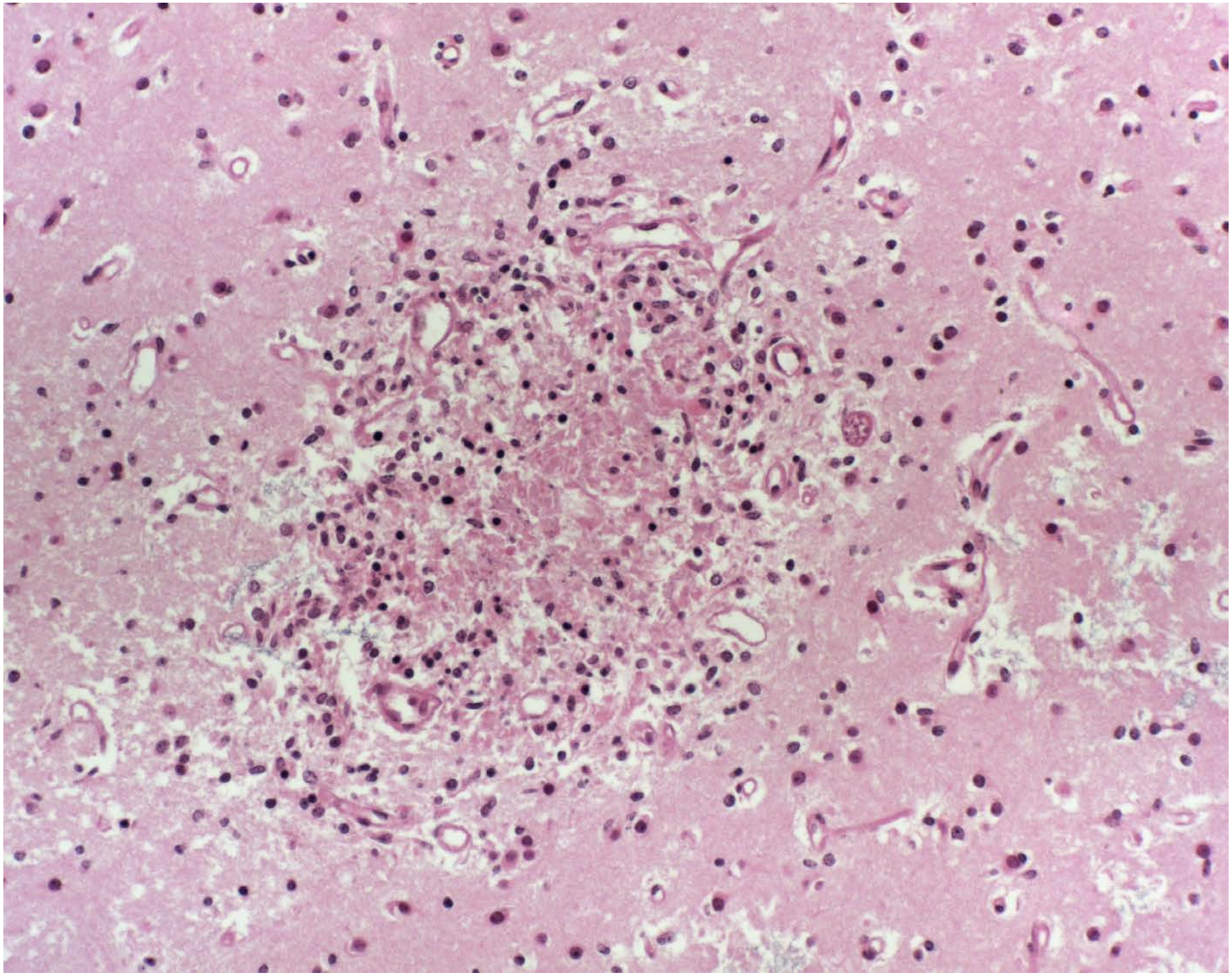
Fig. 8. High magnification photomicrograph of aborted ovine fetal brain illustrating focal necrosis of the neuropil with a surrounding glial cell reaction. The arrow indicates a protozoal cyst which stained positively using a monoclonal antibody directed against *Toxoplasma gondii*. Haematoxylin and eosin stain. Scale bar, 50 μm.

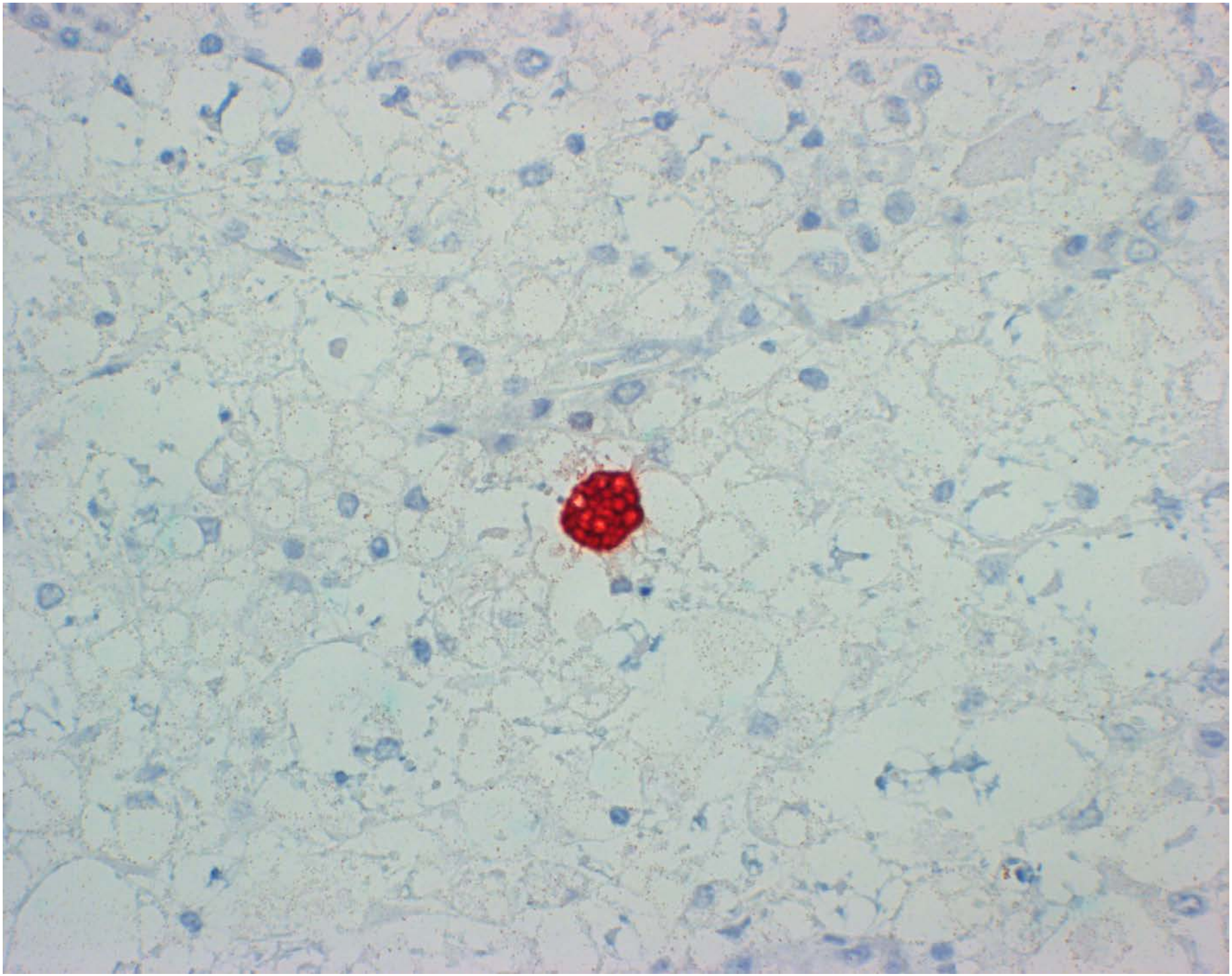
Fig. 9. Photomicrograph illustrating a bright orange-red *Neospora caninum* cyst in the brain of an aborted bovine fetus. Immunohistochemical stain with haematoxylin counterstain. Scale bar, 50 μm.

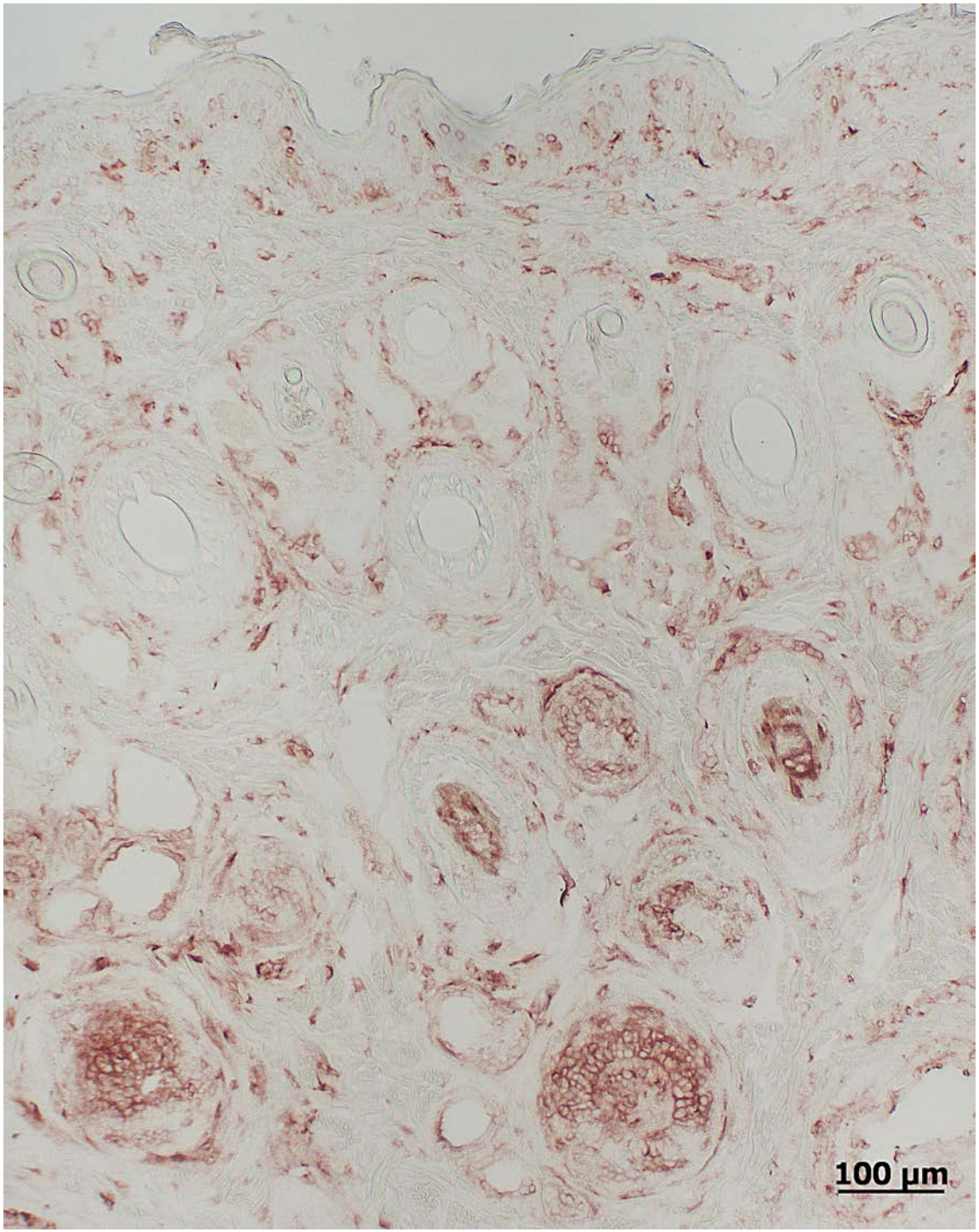
Fig. 10. Snap-frozen tissue sample from the pinna of an aborted bovine fetus persistently-infected with bovine viral diarrhoea virus (BVDV). The skin was labeled immunohistochemically using the anti-BVDV antibody Ca3 at a 1:100 dilution. Hair root sheath and basal epidermal cells, as well as dermal fibrocytes and blood vessels exhibit red-brown granular intra-cytoplasmic virus-positive labeling. EnVision immunohistochemical method without counterstain. Scale bar, 100 μm.










100 µm